



## D E C L A R A T I O N

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conversant with the Japanese language and that I am the  
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best of my knowledge and belief the following is a true and  
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A handwritten signature in black ink, appearing to read "K. Ishii", written over a horizontal line.

Kazuo ISHII

Hei 11-284871

(Translation)

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[TITLE OF DOCUMENT]	Specification	1
[TITLE OF DOCUMENT]	Drawing	1
[TITLE OF DOCUMENT]	Abstract	1
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(Translation)

[DOCUMENT NAME] Specification

[TITLE OF THE INVENTION] Glucose sensor

[CLAIMS]

[Claim 1] A glucose sensor comprising: an electrically insulating base plate; an electrode system including at least a working electrode and a counter electrode formed on said base plate; and a reaction layer containing at least pyrrolo-quinoline quinone dependent glucose dehydrogenase, formed in contact with or in the vicinity of said electrode system, wherein said reaction layer contains at least one kind of additive selected from the group consisting of gluconic acid and salts thereof.

[Claim 2] The glucose sensor in accordance with Claim 1, wherein said reaction layer further contains at least one kind of additive selected from the group consisting of phthalic acid, salts of phthalic acid, maleic acid, salts of maleic acid, succinic acid and salts of succinic acid.

[Claim 3] The glucose sensor in accordance with Claim 1 or 2, wherein said reaction layer further contains calcium ions.

[Claim 4] The glucose sensor in accordance with any of Claims 1 to 3, wherein said salt of gluconic acid is potassium gluconate, sodium gluconate, calcium gluconate, cobalt gluconate, or copper gluconate.

[Claim 5] The glucose sensor in accordance with any of Claims 1 to 4, wherein said reaction layer further contains an electron mediator.

[DETAILED DESCRIPTION OF THE INVENTION]

[0001]

[Technical Field to Which the Invention Belongs]

The present invention relates to a glucose sensor capable of rapidly and simply determining a specific component in a sample with high accuracy. More specifically, the present invention relates to a glucose sensor using pyrrolo-quinoline quinone dependent glucose dehydrogenase.

[0002]

[Prior Art]

Conventionally, a variety of biosensors have been proposed as a system for simply determining a specific component in a sample solution without diluting or stirring the sample solution. As one example of the biosensors, for instance, the following sensor has been known (Japanese Laid-Open Patent Publication No. Hei 2-062952).

This biosensor is fabricated by forming an electrode system comprising a working electrode, a counter electrode and a reference electrode on an electrically insulating base plate by screen printing or other method and forming thereon an enzyme reaction layer comprising a hydrophilic polymer, an oxidoreductase and an electron acceptor in contact with the electrode system.

When a sample solution containing a substrate is dropped on the enzyme reaction layer of this biosensor, the enzyme reaction layer is dissolved, and the substrate and the enzyme react with each other, thereby reducing the electron acceptor. Thereafter, the reduced electron acceptor is electrochemically oxidized, and the concentration of the substrate in the sample solution can be determined from an oxidation current value obtained in this oxidation.

[0003]

According to the biosensor as mentioned above, in theory, it is possible to measure various substances by selecting an enzyme whose substrate is a substance to be measured.

For instance, if glucose oxidase is selected as the enzyme, it is possible to fabricate a glucose sensor for measuring the concentration of glucose in a sample solution.

In the biosensor having the structure as mentioned above, the enzyme is normally retained in the sensor in a dried state. Since the enzyme is mainly composed of protein, if the enzyme is exposed to moisture in the air, etc. over a long period, there is a risk of the denaturation of the enzyme. Moreover, in an extreme case, there is a risk of the inactivation of the enzyme.

For this reason, if the sensor is stored for a long time, the enzyme activity is lowered and the amount of enzyme that reacts with the substrate becomes insufficient, and thus

there is a possibility that the resultant response current value is not proportional to the concentration of the substrate.

Therefore, in order to obtain a biosensor excelling in the storage stability, it is important to provide an environment for retaining the activity of the enzyme for a long time in the vicinity of the enzyme. Moreover, it is necessary to improve the response of the sensor by facilitating smooth movement of the electrons and substrate during an enzyme reaction.

[0004]

On the other hand, in order to fabricate a high-performance glucose sensor, pyrrolo-quinoline quinone dependent glucose dehydrogenase (hereinafter referred to as the "PQQ-GDH") has conventionally been used as the enzyme. In the glucose sensor using the PQQ-GDH, since oxygen is not involved in the catalytic reaction of the PQQ-GDH, this sensor has a characteristic that the enzyme reaction does not receive any effect of dissolved oxygen in blood, etc. Therefore, the measurement value given by this glucose sensor never varies depending on the oxygen partial pressure in the sample solution. In other words, it is possible to obtain a high-performance sensor.

[0005]

[Problem That the Invention Is to Solve]

In a case where the PQQ-GDH is used as the enzyme of

the glucose sensor, however, there has been revealed a problem that the response value is lowered due to storage. The lowering of the response value interferes with the accurate determination of glucose.

In view of such problems, it is an object of the present invention to provide a high-performance glucose sensor having excellent storage stability.

[0006]

[Means for Solving the Problem]

A glucose sensor in accordance with the present invention is a glucose sensor comprises an electrically insulating base plate; an electrode system including at least a working electrode and a counter electrode formed on the base plate; and a reaction layer containing at least pyrrolo-quinoline quinone dependent glucose dehydrogenase, formed in contact with or in the vicinity of the electrode system, and is characterized in that the reaction layer contains at least one kind of additive selected from the group consisting of gluconic acid and salts of gluconic acid.

It is preferred that the reaction layer further contains at least one kind of additive selected from the group consisting of phthalic acid, salts of phthalic acid, maleic acid, salts of maleic acid, succinic acid and salts of succinic acid.

It is preferred that the reaction layer further contains calcium ions.



It is preferred that the salt of gluconic acid is potassium gluconate, sodium gluconate, calcium gluconate, cobalt gluconate, or copper gluconate.

It is preferred that the reaction layer further contains an electron mediator.

[0007]

[Mode for Embodying the Invention]

As described above, a glucose sensor of the present invention is obtained by adding gluconic acid and/or a salt thereof to a reaction layer containing the PQQ-GDH as an enzyme.

The present inventors have found that the storage stability of the sensor can be significantly improved by adding gluconic acid and/or the salt thereof to the reaction layer containing the PQQ-GDH. It is deemed that gluconic acid and/or the salt thereof protects the PQQ-GDH from changes in the environment such as the conditions of temperature, humidity and charge, thereby improving the storage stability. In order to enhance such an effect, it is preferred to form the reaction layer by a method in which a mixed solution of gluconic acid and/or the salt thereof and the PQQ-GDH is dropped to a place where the reaction layer is to be formed and then dried. When the reaction layer is formed according to this method, since the enzyme is surrounded by gluconic acid at a molecular level, it is possible to effectively protect the PQQ-GDH from changes in the environment such as

the conditions of temperature, humidity and charge. As a result, the activity of the enzyme can be stabilized for a long time.

Examples of additives which are expected to produce the above effects include potassium gluconate, sodium gluconate, calcium gluconate, cobalt gluconate and copper gluconate as well as gluconic acid. In particular, when potassium gluconate is used, it is possible to obtain a glucose sensor having excellent storage stability and response characteristic and a very low blank value. Here, the blank value is a sensor response value obtained by the use of a sample solution containing no glucose as a substrate, for example, water.

Although phthalic acid, maleic acid, succinic acid and the salts thereof are not as good as gluconic acid and salts of gluconic acid when used alone, since they have an effect of protecting the PQQ-GDH, if they are added together with gluconic acid or the salt thereof, it is possible to further improve the storage stability of the sensor by the synergistic effect.

Since the additives as above mentioned are easily dissolved in water, if they are contained in the reaction layer, when the sample solution is added to the reaction layer, the reaction layer is immediately dissolved in the sample solution and the enzyme reaction and electrode reaction can proceed smoothly, which is advantageous.

[0008]

Phthalic acid, maleic acid, succinic acid and the salts thereof are all compounds that can be used as a buffer, and may be added to the reagent for forming the reaction layer by adjusting them to a predetermined pH with acid such as hydrochloric acid and acetic acid or alkali such as NaOH and KOH, if necessary. A suitable pH is between 5.0 and 8.5. Of course, compounds obtained by adding these additives to other buffer may be used.

In a disposable type sensor for measuring 0.5 to 5  $\mu$ l blood as a sample solution, the amount of gluconic acid or the salt thereof to be added may be within the range of 1.5 to 150  $\mu$ g/sensor with respect to the amount of enzyme of 0.2 to 20 U/sensor, and is preferably between 15 and 50  $\mu$ g/sensor from the viewpoint of the storage stability and a reduction of the blank value. Meanwhile the amount of phthalic acid, maleic acid, succinic acid and the salts thereof to be added is preferably between 0.25 and 2.5  $\mu$ g/sensor with respect to the above-mentioned sensor. Here, U represents unit.

An example of other preferable additive is calcium chloride that gives calcium ions. In general, calcium ions are necessary when the PQQ-GDH forms a dimer. Therefore, when calcium ions are introduced into the reagent for forming the reaction layer by calcium chloride, etc., it is possible to prevent dissociation of the PQQ-GDH to a dimer during or after the fabrication of the sensor, and therefore the calcium ions

are useful for retaining the activity of PQQ-GDH. The amount of calcium chloride to be added is preferably between 5 and 70 ng (nanogram)/sensor with respect to the above-mentioned sensor.

[0009]

It is preferred that the reaction layer of the biosensor of the present invention contains an electron mediator which is reduced with the enzyme reaction. For this electron mediator, it is possible to use potassium ferricyanide, p-benzoquinone and derivatives thereof, phenazine methosulphate, methylene blue, ferrocene and derivatives thereof, etc.

The reaction layer of the biosensor of the present invention may contain a hydrophilic polymer. By adding the hydrophilic polymer to the reaction layer, it is possible to prevent separation of the reaction layer from the electrode system surface or the base plate surface. Moreover, since the hydrophilic polymer has the effect of preventing cracks in the reaction layer surface, it is effective for an increase of the reliability of the biosensor.

As such a hydrophilic polymer, it is possible to suitably use carboxymethyl cellulose, hydroxyethyl cellulose, hydroxypropyl cellulose, methyl cellulose, ethyl cellulose, ethyl hydroxyethyl cellulose, carboxymethylethyl cellulose, polyvinyl pyrrolidone, polyvinyl alcohol, polyamino acid such as polylysine, polystyrene sulphonate, g

elatin and derivatives thereof, polymers of acrylic acid and salts thereof, polymers of methacrylic acid and salts thereof, starch and derivatives thereof, polymers of maleic anhydride and salts thereof, agarose gel and derivatives thereof.

[0010]

The reaction layer in the biosensor may be placed at various positions as well as on the electrode system formed on the electrically insulating base plate if it does not impair the effects of the present invention. For example, it is possible to place the reaction layer at a position other than on the electrode system of the base plate. Moreover, the biosensor preferably includes a cover member. This cover member is combined with the base plate to form a sample solution supply path between the cover member and the base plate, for supplying the sample solution to the electrode system. It is possible to position the reaction layer on this cover member's face exposed to the sample solution supply path.

As the method for measuring a current for oxidizing the electron mediator reduced with the enzyme reaction, there are two types of methods: a two-electrode method using only a working electrode and a counter electrode; and a three-electrode method further comprising a reference electrode, and the three-electrode method enables more accurate measurement.

[0011]

Here, for the reaction layer of the biosensor of the

present invention, in addition to the above-mentioned additives, it is possible to add other stabilizer unless it impairs the effects of the present invention. Examples of such a stabilizer include metallic salts, proteins, amino acids, sugars, organic acids, and surface active agents.

Examples of metallic salt may include halides such as strontium and manganese, the sulfates and nitrites thereof.

Preferred proteins are ones that do not affect the enzyme activity, and examples of such proteins include bovine serum albumin (BSA), egg albumin, and gelatin.

As the amino acid, it is possible to use glycylglycine, polylysine, etc. as well as typical amino acids such as lysine, histidine and glutamic acid. Among them, highly water-soluble amino acids are preferable.

[0012]

As the sugar, it is possible to use any kinds of sugars, such as monosaccharide, disaccharide, oligosaccharide and polysaccharide. It is also possible to use derivatives thereof. More specifically, examples of sugars include glucose, fructose, galactose, mannose, xylose, sucrose, lactose, maltose, trehalose, maltotriose, maltocyclcyclodextrin,  $\alpha$ -cyclodextrin,  $\beta$ -cyclodextrin,  $\gamma$ -cyclodextrin, dextrin, amylose, glycogen, starch, inulin, glucosamine, inositol, mannitol, sorbitol, ribitol and deoxyglucose.

Examples of the organic acid include  $\alpha$ -ketoglutaric acid, malic acid, fumaric acid, cholic acid, and deoxycholic

acid.

As the surface active agent, it is preferred to use a nonionic surface active agent.

[0013]

In addition, boric acid, borax, potassium chloride, sodium chloride, ammonium sulfate, glycerol, Ficoll, EDTA, EGTA, DTT, DTE, GSH, 2-mercaptoethanol, etc. may be added.

The amount of these stabilizers to be added is preferably between 0.0001 and 1.0 part by weight based on 1.0 part by weight of glucose dehydrogenase.

The pyrrolo-quinoline quinone dependent glucose sensor of the present invention that contains the above-mentioned additives and further contains the above-mentioned stabilizers, if necessary, can retain the performance thereof at low costs without viciously affecting the basic performance of the enzyme.

As pyrrolo-quinoline quinone as a coenzyme to be used in the present invention, it is possible to use one from any source.

[0014]

#### [Working Examples]

In the following, some examples will be used to explain the present invention, but the present invention is not necessarily limited to only these examples.

FIG. 1 is an exploded perspective view of a biosensor in accordance with one example of the present

invention, omitting the reaction layer. A silver paste is printed on an electrically insulating base plate 1 made of polyethylene terephthalate by screen printing to form leads 2 and 3. Subsequently, a conductive carbon paste containing a resin binder is printed on the base plate 1 to form a working electrode 4. This working electrode 4 is in contact with the lead 2. Further, an insulating paste is printed on the base plate 1 to form an insulating layer 6. The insulating layer 6 covers the peripheral portion of the working electrode 4, so that the area of the exposed portion of the working electrode 4 is kept constant. Next, a ring-shaped counter electrode 5 is formed by printing a conductive carbon paste containing a resin binder on the base plate 1 so as to be in contact with the lead 3.

[0015]

After forming a reaction layer on the insulating base plate 1 in a later-described manner, a spacer 8 including a slit 10 and a cover 9 having an air bent 11 adhere to each other in a positional relationship as shown by the dashed lines of FIG. 1, thereby fabricating the biosensor. A sample solution supply path is formed in the portion of the slit 10 of the spacer 8. The open end of the slit 10 at an end portion of the sensor serves as the sample supply port to the sample solution supply path.

FIG. 2 is a vertical cross sectional view of the biosensor of the present invention. A reaction layer 7



containing an enzyme and an electron mediator is formed on the base plate 1 on which the electrode system is formed. The reaction layer 7 is preferably formed on the electrode system, but it may be formed in the vicinity of the electrode system, for example, on the cover side so that it is exposed to the sample solution supply path. In the illustrated example, the reaction layer 7 is composed of a hydrophilic polymer layer 7a and a layer 7b which contains the PQQ-GDH and additives and is formed on the hydrophilic polymer layer 7a.

[0016]

«Comparative Example 1»

5  $\mu$ l of a 0.5 wt% aqueous solution of sodium salt of carboxymethyl cellulose (hereinafter abbreviated to "CMC") as a hydrophilic polymer was dropped onto the electrode system of the base plate 1 of FIG. 1 and dried in a 50°C hot-air drier for 10 minutes to form a CMC layer 7a. Subsequently, 5  $\mu$ l of a mixed aqueous solution containing 1000 U/ml of PQQ-GDH and 50  $\mu$ M of potassium ferricyanide was dropped onto the CMC layer 7(sic) and dried to form a layer 7b. A glucose sensor was fabricated in such a manner.

Next, as a sample solution, blood conditioned to have a glucose concentration of 30 to 620 mg/dl was prepared. Then, this sample solution was dropped onto the reaction layer 7. When the sample solution containing glucose is supplied to the reaction layer, glucose in the sample is oxidized by the PQQ-GDH. Then, at the same time as the oxidation, potassium

ferricyanide in the reaction layer is reduced to potassium ferrocyanide. Here, 30 seconds after the dropping of the sample solution, a voltage of +0.5 V was applied to the working electrode 4 on the basis of the counter electrode 5 so as to oxidize potassium ferrocyanide. Then, 5 seconds later, the value of a current flowing across the counter electrode and the working electrode was measured.

[0017]

The current value was measured for blood conditioned for a variety of glucose concentrations, and the response characteristic graph of the sensor was produced by plotting the glucose concentration in the horizontal axis and the current value in the vertical axis. The results are shown by the solid line in FIG. 3.

A biosensor fabricated in the same manner was stored for one week at 40°C, and then the response characteristic graph of this biosensor was produced. The results are shown by the dotted line in FIG. 3.

It is found from FIG. 3 that there is a certain correlation between the glucose concentration and the response current value. However, it is found that the response characteristic of the sensor stored for one week at 40°C was lowered in comparison with the sensor immediately after the fabrication.

[0018]

«Example 1»

After forming the CMC layer 7a in the same manner as in Comparative Example 1, 5  $\mu$ l of a mixed aqueous solution containing 1000 U/ml of PQQ-GDH, 50  $\mu$ M of potassium ferricyanide and 40 mM of potassium gluconate was dropped onto the CMC layer 7a and dried to form the layer 7b. A glucose sensor was fabricated in such a manner.

Next, in the same manner as in Comparative Example 1, the response characteristic graph was produced for the sensor immediately after the fabrication and the sensor after being stored for one week at 40°C. The results are shown by the solid line and the dotted line, respectively, in FIG. 4.

It is found from FIG. 4 that there is a certain correlation in the obtained sensor. It is found that, by a comparison with the sensor immediately after the fabrication, the sensor after the one-week storage at 40°C had a smaller lowering in the response, particularly in a range of not less than 400 mg/dl. It is thereby found that the storage characteristic of the glucose sensor is significantly improved by the addition of potassium gluconate.

[0019]

#### 《Example 2》

After forming the CMC layer 7a in the same manner as in Comparative Example 1, 5  $\mu$ l of a mixed aqueous solution containing 1000 U/ml of PQQ-GDH, 50  $\mu$ M of potassium ferricyanide, 40 mM of potassium gluconate and 20  $\mu$ M of potassium hydrogen phthalate was dropped onto the CMC layer 7a

and dried to form the layer 7b. A glucose sensor was fabricated in such a manner.

Next, in the same manner as in Comparative Example 1, the response characteristic graph was produced for the sensor immediately after the fabrication and the sensor after being stored for one week at 40°C. The results are shown by the solid line and the dashed line, respectively, in FIG. 5.

It is found from FIG. 5 that there is a certain correlation in the obtained sensor. It is found that there is almost no difference in response characteristic between the sensor immediately after the fabrication and the sensor after the one-week storage at 40°C, and that the storage characteristic of the obtained sensor was thus improved.

[0020]

#### 《Example 3》

After forming the CMC layer 7a in the same manner as in Comparative Example 1, 5  $\mu$ l of a mixed aqueous solution containing 1000 U/ml of PQQ-GDH, 50  $\mu$ M of potassium ferricyanide, 40 mM of potassium gluconate and 20  $\mu$ M of maleic acid was dropped onto the CMC layer 7a and dried to form the layer 7b. A glucose sensor was fabricated in such a manner.

Next, in the same manner as in Comparative Example 1, the response characteristic graph was produced for the sensor immediately after the fabrication and the sensor after being stored for one week at 40°C. The results are shown by the solid line and the dotted line, respectively, in FIG. 6.

It is found from FIG. 6 that there is a certain correlation in the obtained sensor. It is found that there is almost no difference in response characteristic between the sensor immediately after the fabrication and the sensor after the one-week storage at 40°C, and that the storage characteristic of the obtained sensor was thus improved.

[0021]

《Example 4》

After forming the CMC layer 7a in the same manner as in Comparative Example 1, 5  $\mu$ l of a mixed aqueous solution containing 1000 U/ml of PQQ-GDH, 50  $\mu$ M of potassium ferricyanide, 40 mM of potassium gluconate and 20  $\mu$ M of succinic acid was dropped onto the CMC layer 7a and dried to form the layer 7b. A glucose sensor was fabricated in such a manner.

Next, in the same manner as in Comparative Example 1, the response characteristic graph was produced for the sensor immediately after the fabrication and the sensor after being stored for one week at 40°C. The results are shown by the solid line and the dotted line, respectively, in FIG. 7.

It is found from FIG. 7 that there is a certain correlation in the obtained sensor. It is found that there is almost no difference in response characteristic between the sensor immediately after the fabrication and the sensor after the one-week storage at 40°C, and that the storage characteristic of the obtained sensor was thus improved.

[0022]

《Example 5》

After forming the CMC layer 7a in the same manner as in Comparative Example 1, 5  $\mu$ l of a mixed aqueous solution containing 1000 U/ml of PQQ-GDH, 50  $\mu$ M of potassium ferricyanide, 40 mM of potassium gluconate, 20  $\mu$ M of potassium hydrogen phthalate and 75  $\mu$ M of calcium chloride was dropped onto the CMC layer 7a and dried to form the layer 7b. A glucose sensor was fabricated in such a manner.

Next, in the same manner as in Comparative Example 1, the response characteristic graph was produced for the sensor immediately after the fabrication and the sensor after being stored for one week at 45°C. The results are shown by the solid line and the dotted line, respectively, in FIG. 8.

It is found from FIG. 8 that there is a certain correlation in the obtained sensor. It is found that there is almost no difference in response characteristic between the sensor immediately after the fabrication and the sensor after the one-week storage at 45°C, and that the obtained sensor exhibits an excellent storage characteristic under a high-temperature storage condition of 45°C for one week.

[0023]

[Effects of the Invention]

According to the present invention, as described above, it is possible to obtain a high-performance glucose sensor having excellent storage stability.

[BRIEF EXPLANATION OF THE DRAWINGS]

[FIG. 1]

A perspective view of a glucose sensor in accordance with one example of the present invention, omitting a reaction layer.

[FIG. 2]

A vertical cross-sectional view of the vital part of the glucose sensor shown in FIG. 1.

[FIG. 3]

A graph showing the response characteristics of a glucose sensor produced as a comparative example of the present invention.

[FIG. 4]

A graph showing the response characteristics of a glucose sensor in one example of the present invention.

[FIG. 5]

A graph showing the response characteristic of a glucose sensor in another example of the present invention.

[FIG. 6]

A graph showing the response characteristic of a glucose sensor in yet another example of the present invention.

[FIG. 7]

A graph showing the response characteristic of a glucose sensor in another example of the present invention.

[FIG. 8]

A graph showing the response characteristic of a

glucose sensor of the present invention.

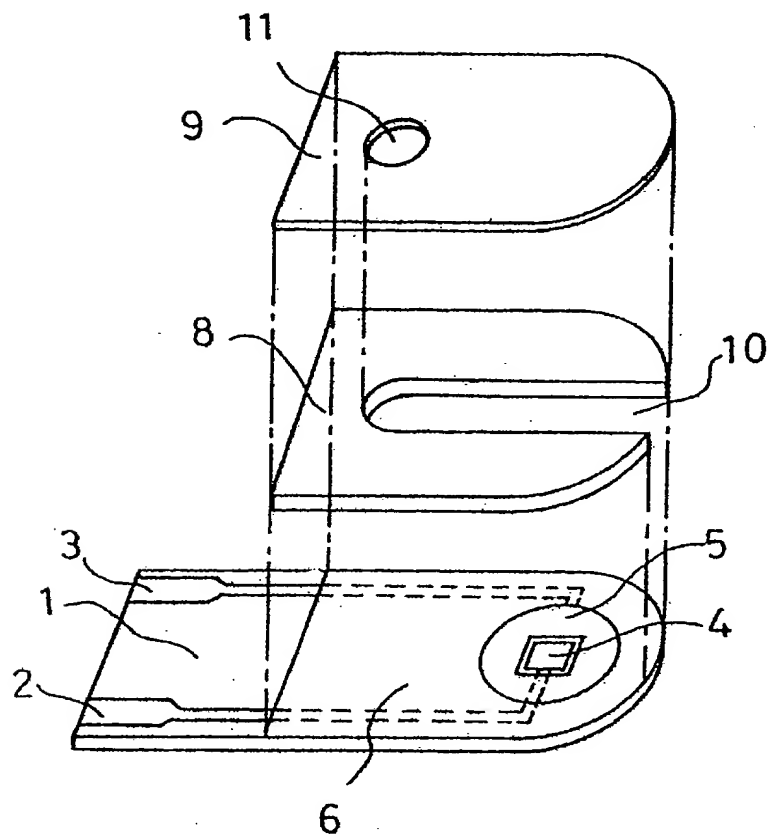
[Explanation of Reference Numerals]

1. Electrically insulating base plate
- 2, 3 Lead
4. Working electrode
5. Counter electrode
6. Insulating layer
7. Reaction layer
- 7a. Hydrophilic polymer layer
- 7b. Layer containing PQQ-GDH
8. Spacer
9. Cover
10. Slit
11. Air bent

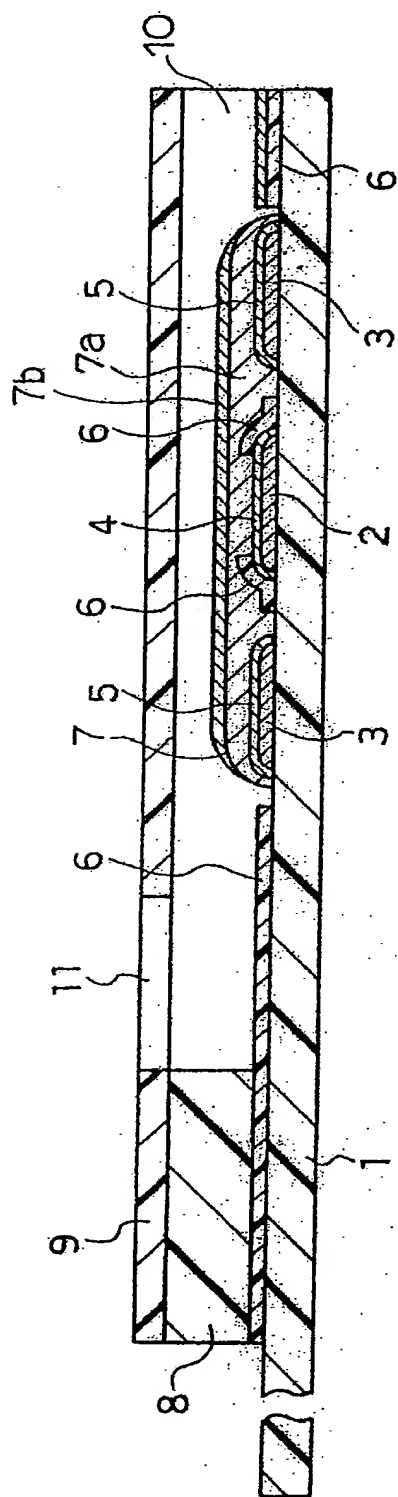


【書類名】 図面 [DOCUMENT NAME] Drawings

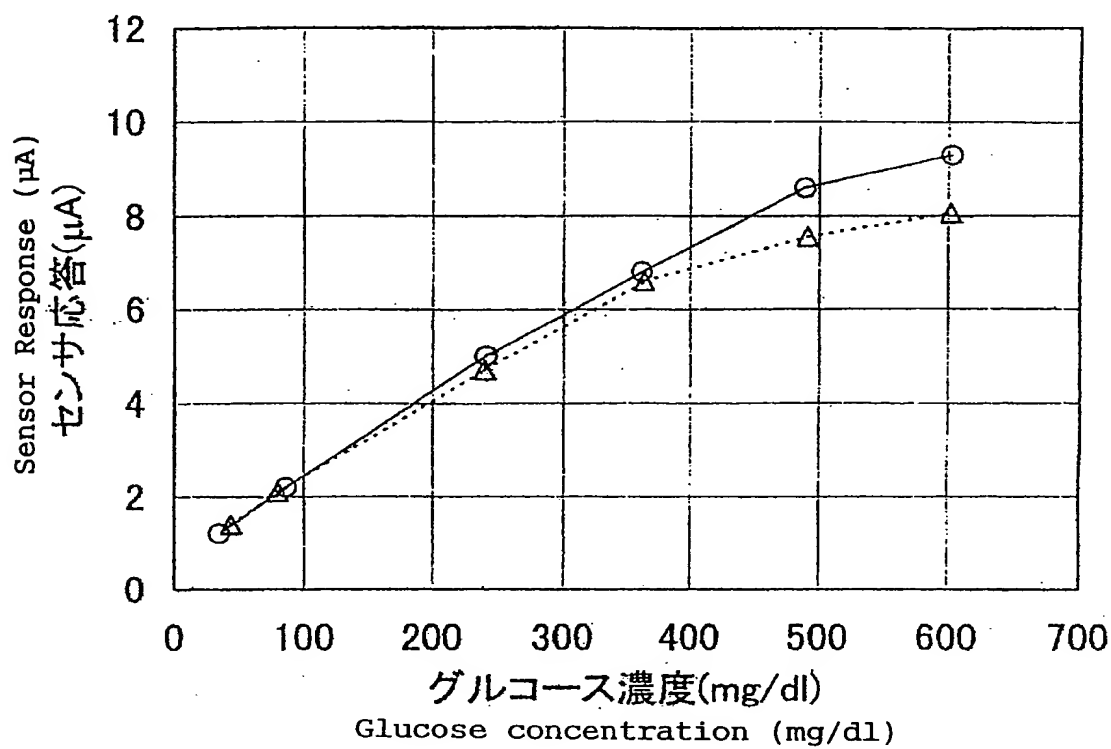
【図1】 [FIG. 1]



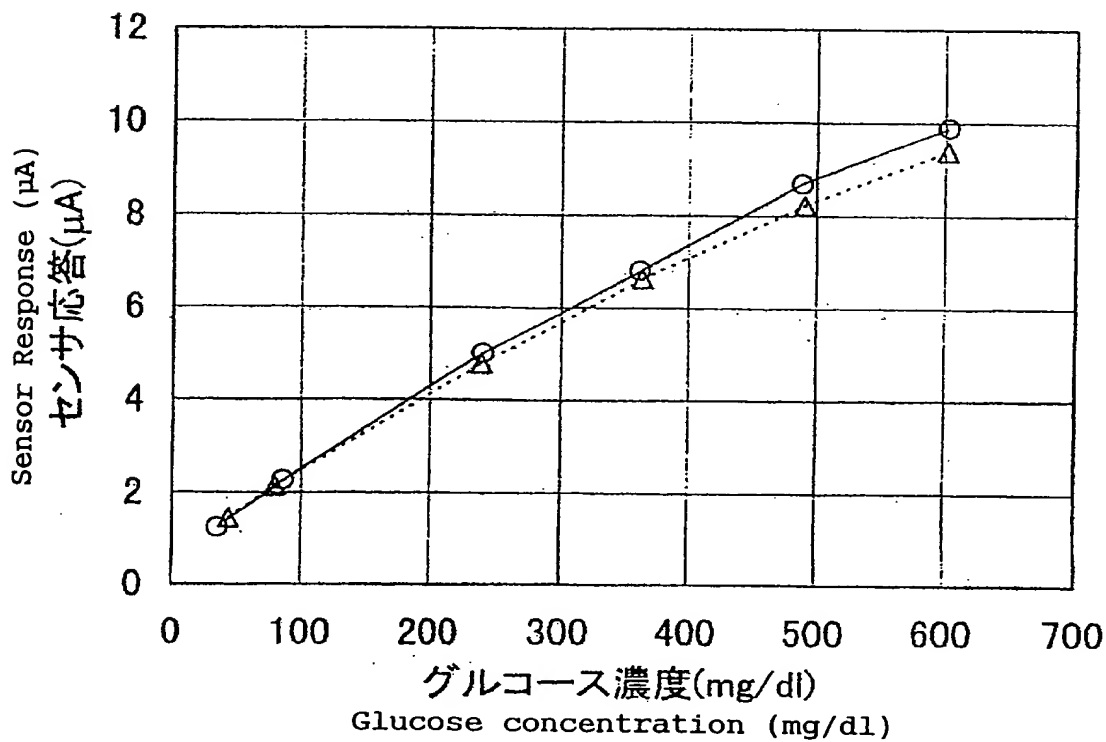
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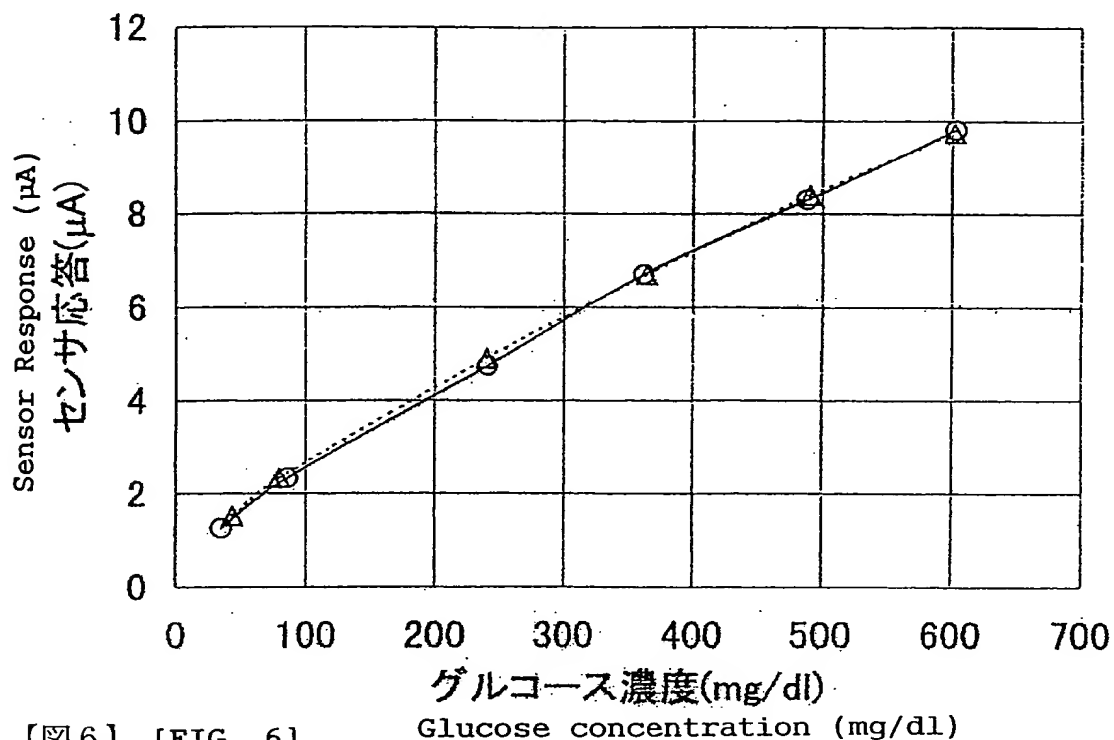
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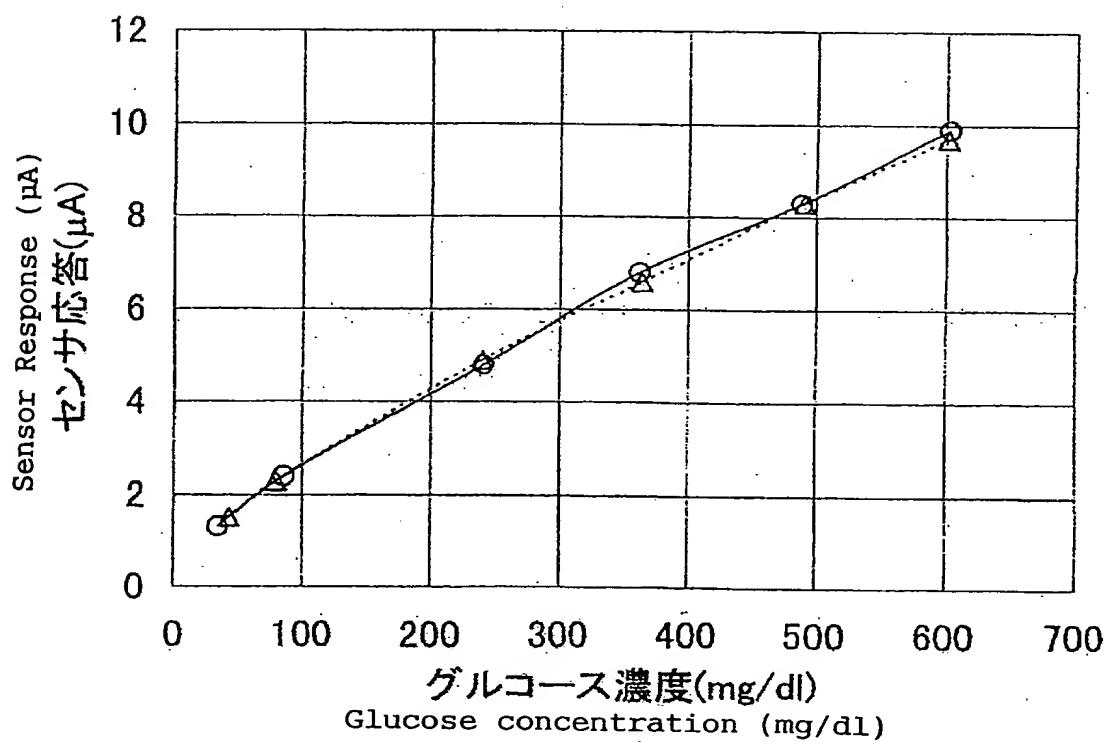
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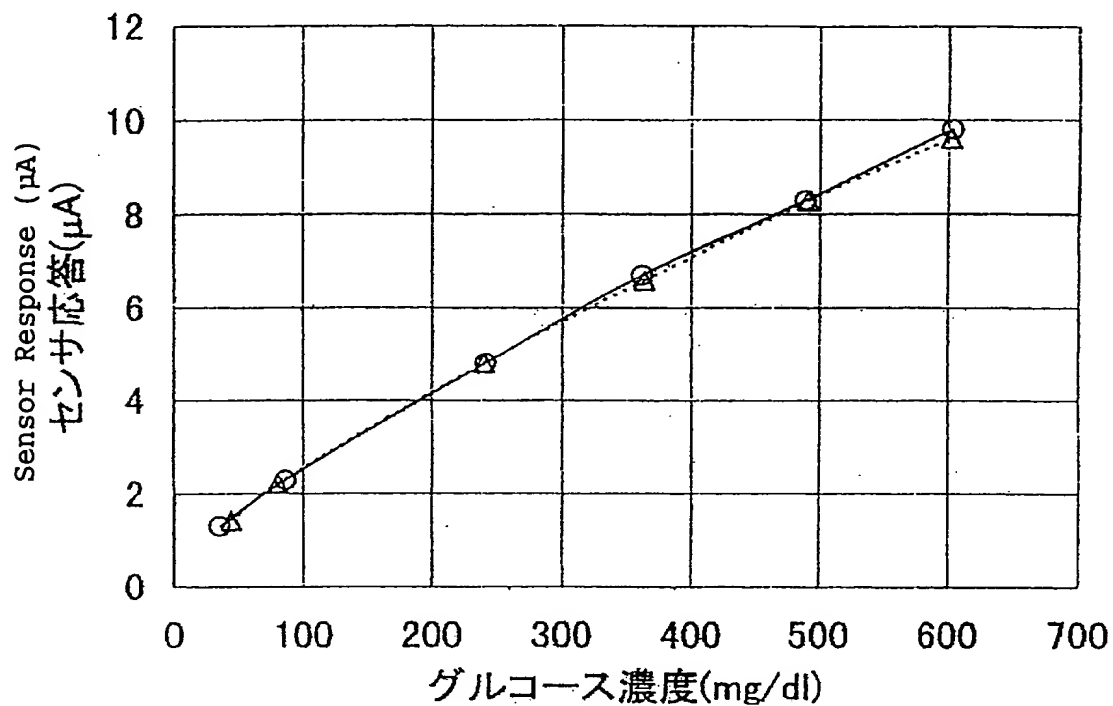
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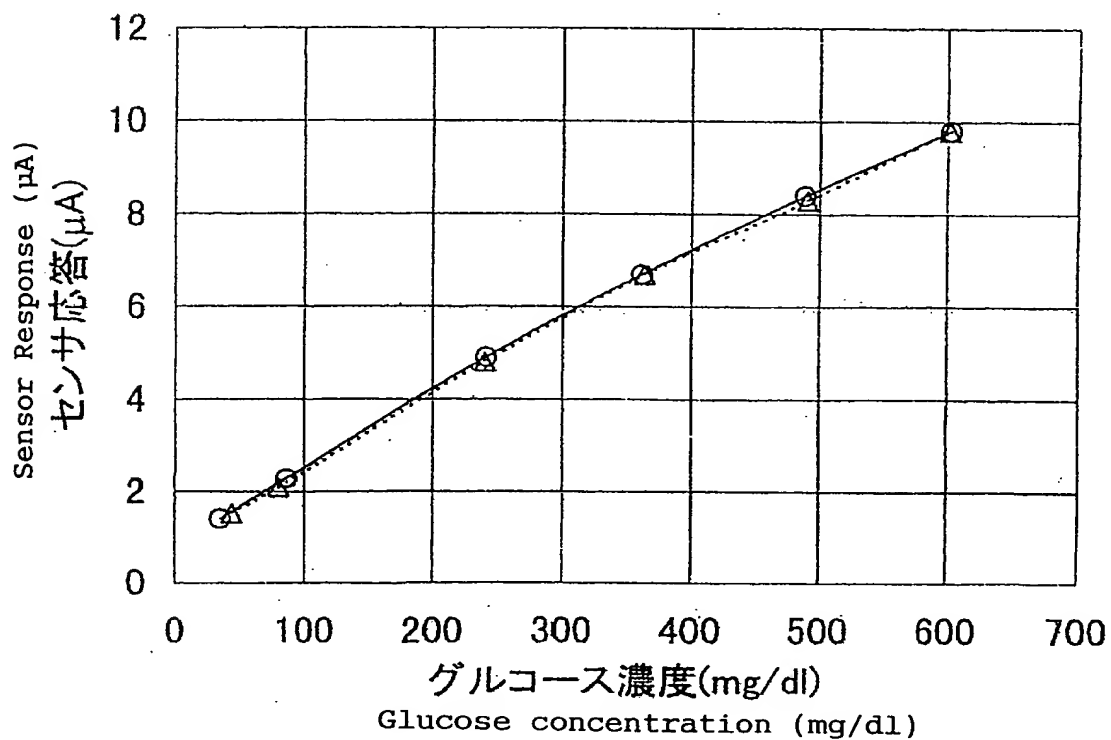
【図6】 [FIG. 6]



【図 7】 [FIG. 7]



【図 8】 [FIG. 8]



Hei 11-284871

(Translation)

[DOCUMENT NAME] Abstract

[ABSTRACT]

[OBJECTIVE] A high-performance glucose sensor having excellent storage stability is provided.

[SOLVING MEANS] A sensor comprising: an electrically insulating base plate; an electrode system including at least a working electrode and a counter electrode formed on the base plate; and a reaction layer containing at least pyrrolo-quinoline quinone dependent glucose dehydrogenase, formed in contact with or in the vicinity of the electrode system, wherein the reaction layer contains at least one kind of additive selected from the group consisting of gluconic acid and salts thereof.

[SELECTED DRAWING] None